Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis
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Role of 1,N^2-propanodeoxyguanosine adducts as endogenous DNA lesions in rodents and humans

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Results obtained in a number of studies *in vitro* and *in vivo* support the hypothesis that short- and long-chain enals and their epoxides derived from oxidized polyunsaturated fatty acids are potential endogenous sources of cyclic propano and etheno DNA adducts. We previously reviewed the evidence from some of these studies. Here, we describe the results of our more recent studies on the role of 1,N^2-propanodeoxyguanosine adducts as endogenous DNA lesions. These studies include: the detection of distinct patterns of such adducts in various tissues of different species; the detection of long-chain *trans*-4-hydroxynonenal-derived deoxyguanosine adducts *in vivo*; the specificity of the formation of enal-derived propano adducts from ω–3 and ω–6 polyunsaturated fatty acids; and the detection of acrolein- and crotonaldehyde-derived adducts in human oral tissue DNA and their increased levels in smokers. Taken together, these studies further strengthen the hypothesis that enals produced by lipid peroxidation are the primary source for cyclic propano adducts *in vivo*, but these results cannot rule out the possible contribution of environmental and other sources. The mutagenicity of enals and their epoxides and the results of site-specific mutagenesis studies indicate that the cyclic adducts are potential promutagenic lesions; however, only circumstantial evidence is currently available for their role in carcinogenesis.

Introduction

The propano, etheno and malondialdehyde-derived adducts, named according to their newly adducted ring moeity, are perhaps the most widely studied of the cyclic DNA bases. These adducts are products of the reactions of bi-functional electrophilic compounds with DNA bases. The propano adducts are formed by reactive α,β-unsaturated aldehydes or enals, such as acrolein, crotonaldehyde and *trans*-4-hydroxy-2-nonenenal (HNE) (Chung *et al.*, 1984; Winter *et al.*, 1986; Meerman *et al.*, 1989; Eder & Hoffman, 1993; Yi *et al.*, 1997). Acrolein and crotonaldehyde are ubiquitous pollutants in the environment and also products of peroxidation of lipids, whereas the third is a unique oxidation product of ω–6 polyunsaturated fatty acids (Esterbauer *et al.*, 1991; Wu & Lin, 1995). The etheno adducts are products of reactions with chloroacetaldehyde, 1-substituted oxiranes and the epoxides of enals (Sodum & Chung, 1991; Guengerich *et al.*, 1993; Park *et al.*, 1993). The malondialdehyde-derived adduct is formed either by reaction with malondialdehyde, a known product of lipid peroxidation, or with base propenals arising from oxidized DNA (Marnett *et al.*, 1986; Dedon *et al.*, 1998).

In the past several years, we have studied acrolein-, crotonaldehyde- and HNE-derived 1,N^2-propanodeoxyguanosine (acrolein-, crotonaldehyde- and HNE-dG; Figure 1) and related cyclic adducts. We showed by a ^{32}P-postlabelling–high-performance liquid chromatography (HPLC) assay that the cyclic propano dG adducts are present in tissue DNA of humans and untreated rodents at relatively high levels (Nath & Chung, 1994; Nath *et al.*, 1994, 1996). These observations raised the possibility that the adducts are formed *via* endogenous pathways. Because peroxidation of polyunsaturated fatty acids is known to yield enals of various chain lengths, ranging from acrolein to HNE as secondary products (Esterbauer *et al.*, 1991; Wu & Lin, 1995), we proposed that membrane fatty

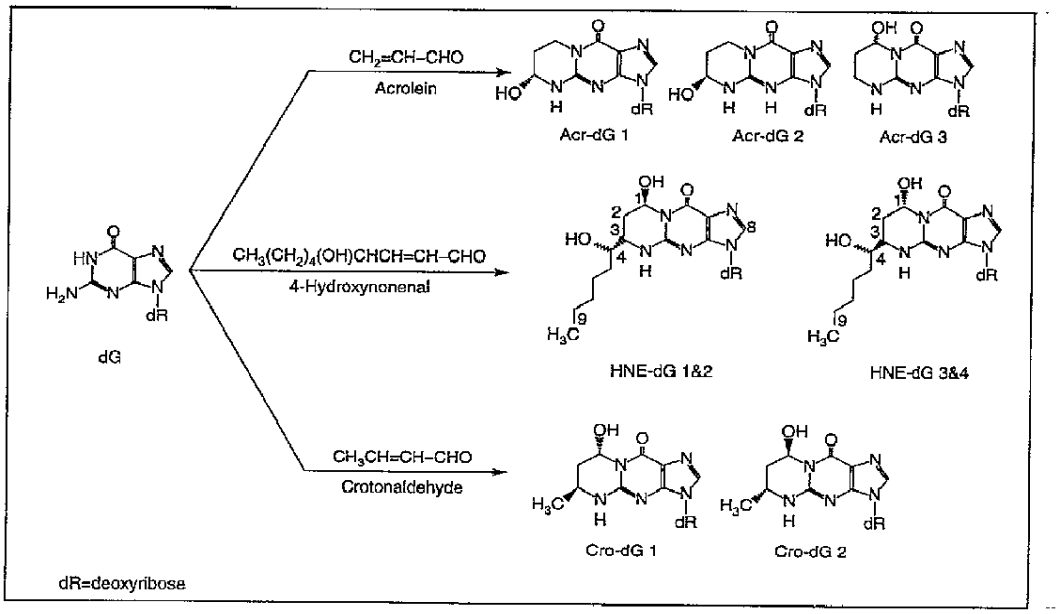

Figure 1. Structures of acrolein–deoxyguanosine (Acr-dG), crotonaldehyde–deoxyguanosine (Cro-dG) and *trans*-4-hydroxy-2-nonenenal–deoxyguanosine (HNE-dG) adducts

Acr-dG consists of three isomers, and one of them, Acr-dG 3 with a hydroxy group adjacent to N1 of guanine, is the predominant one detected *in vivo*, Cro-dG consists of two isomers, and HNE-dG consists of four isomers. The alkyl and hydroxy group of the propano ring are in *trans* configuration in Cro-dG and HNE-dG (Chung *et al.*, 1984; Yi *et al.*, 1997).

acids may be the endogenous sources of these cyclic adducts. We carried out a number of studies *in vitro* and *in vivo* to determine whether oxidation of polyunsaturated fatty acids is indeed the source. These studies show that:

- The levels of propano adducts are dramatically increased in rat liver DNA after depletion of glutathione.
- Their levels are also increased in the liver DNA of carbon tetrachloride-treated rats and of a mutant strain of Long Evans rats genetically predisposed to a higher level of lipid peroxidation.
- The cyclic propano-dG adducts of acrolein and crotonaldehyde are background lesions which appear to occur with distinct patterns in various tissues of several species, including humans.
- In addition to the propano adducts derived from the short-chain enals, the HNE-dG adducts are also present *in vivo* as background lesions.
- Under conditions of stimulated lipid peroxidation *in vitro*, acrolein-, crotonaldehyde- and HNE-dG adducts are formed specifically from arachidonic acid, linoleic acid (ω–6) or docosahexaenoic acid (ω–3).
- Acrolein- and crotonaldehyde-dG are present in human oral tissue, and their levels are increased in smokers.

Collectively, these results indicate that oxidized polyunsaturated fatty acids are an important endogenous source of propano adducts. We have reviewed some of these studies previously (Chung *et al.*, 1996). Here, we describe recent studies and discuss the possible roles of these adducts in mutagenesis and carcinogenesis.

Table
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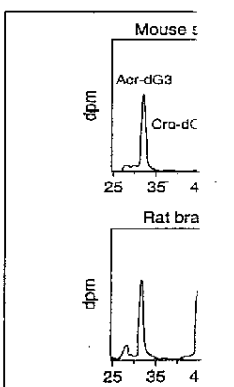

Figure 2. Presence of sp
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chromatography (HPLC)

The retention times varie

Table 1. Species and tissues shown to contain acrolein-deoxyguanosine and crotonaldehyde–deoxyguanosine adducts and the range of total adduct levels in each species

Species	Strain	Tissue	Total adduct levels (μmol/mol guanine)
Mouse	A/J	Liver and skin	0.31–1.69
Rat	Fischer 344	Liver, mammary gland, colonic mucosa, kidney, brain, lung, prostate and leukocytes	0.11–1.12
	Wistar	Liver and urinary bladder	
Human		Liver, leukocytes, gingiva and breast	0.01–7.53[a]

[a] The highest levels were found in the gingival DNA of a smoker (Nath *et al.*, 1998)

Presence of acrolein–deoxyguanosine and crotonaldehyde–deoxyguanosine in distinct patterns in tissues and of *trans*-4-hydroxy-2-nonenenal–deoxyguanosine *in vivo*

We have found that acrolein-dG and crotonaldehyde-dG are prevalent DNA lesions in a variety of rodents and human tissues, including colon, lung, brain, prostate, kidney, mammary gland, liver and leukocytes (Nath & Chung, 1994; Nath *et al.*, 1996). Table 1 summarizes the species and tissues in which these adducts have been detected and the range of levels of acrolein-dG plus crotonaldehyde-dG in each species. These adducts are detected in each tissue in distinct patterns (Figure 2). For example, the total levels were the highest in rat brain and lung, followed by

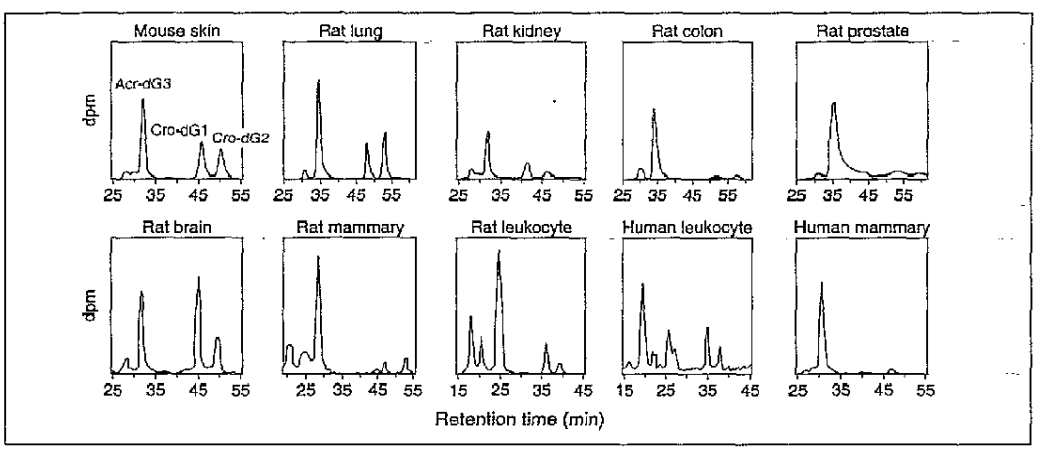

Figure 2. Presence of specific patterns of acrolein–deoxyguanosine and crotonaldehyde–deoxyguanosine adducts in rodent and human tissues in final analysis based on co-migration with the adduct standards on reverse-phase high-performance liquid chromatography (HPLC)

The retention times varied in some cases due to ageing of the HPLC columns.

colon and prostate; the adduct levels in rat kidney were the lowest of all the tissues examined so far. Rat colon, prostate and mammary gland DNA showed almost exclusively acrolein-dG3, an isomer of the acrolein-dG adduct, with very low levels of crotonaldehyde-dG. In contrast, brain and lung had relatively high levels of crotonaldehyde-dG adducts. Human tissues such as the liver and breast consistently showed much greater variation in acrolein-dG and crotonaldehyde-dG levels than mouse and rat tissues, indicating greater individual variation among humans than among inbred animals. While the significance of the tissue-specific and stereoselective pattern of these adducts in tissues is as yet unknown, these results support their endogenous origins and argue against their being artefacts. Furthermore, the levels detected in each tissue may represent a steady state as a result of constant formation and removal. Such a state of equilibrium could be altered by a number of factors, such as antioxidant status, repair activity and, possibly, fatty acid composition in the tissue.

Recently, we detected the long-chain HNE-dG adducts in human and rat tissue by a ^{32}P-postlabelling–HPLC method similar to that for acrolein- and crotonaldehyde-dG. HNE-dG adducts consist of two pairs of diastereomers of HNE-dG, 1,2 and 3,4, because of the chiral carbon in the side chain (Figure 1; Yi *et al.*, 1997). HNE-dG adducts with a long alkyl side-chain are considerably more lipophilic than acrolein-dG or crotonaldehyde-dG, and therefore solid-phase extraction can be used to enrich HNE-dG adducts in DNA hydrolysates, followed by one-dimensional thin-layer chromatography purification, and subsequent analysis by reversed-phase HPLC. The final HPLC analysis of DNA obtained from humans and rat liver or colon showed radioactive peaks due to the DNA in these tissues co-migrating with the ultraviolet standards of HNE-dG 1,2 and 3,4. In addition, the identities of these adducts were confirmed by the co-migration of the 5′-monophosphates of HNE-dG after hydrolysis by polynucleotide kinase. Unlike acrolein and crotonaldehyde, which are ubiquitous environmental pollutants, HNE is a unique product of oxidized polyunsaturated fatty acids. Thus, the detection of HNE-dG adducts indicates that peroxidation of fatty acids is likely to be an important pathway and source for their formation. While the protein adducts of HNE have been detected in tissues (Yoritaka *et al.*, 1996), our study is the first to show the presence of the HNE–DNA adducts in human and rodent tissues *in vivo*.

Cyclic propano adducts are formed from arachidonic acid, linoleic acid and docosahexaenoic acid *in vitro* under lipid peroxidation conditions

In these studies, we examined whether acrolein-dG, crotonaldehyde-dG and HNE-dG are formed when lipid peroxidation is stimulated and whether the formation of each adduct is dependent on the types of polyunsaturated fatty acids. Incubations were carried out with a mixture containing 1.5 mmol/L linoleic acid, arachidonic acid or docosahexaenoic acid and deoxyguanosine 5′-monophosphate (25 µmol or 25 µmol/L) in the presence of $FeSO_4$ (0.75 mmol/L) and NADPH (0.1 mmol/L) in 1 mL Tris-HCl buffer (0.1 mol/L, pH 7.1) under a previously described condition in which the etheno adducts of adenine and cytosine were detected with rat liver microsomes (El Ghissassi *et al.*, 1995). The incubation mixture was analysed by a sequential reversed-phase HPLC for acrolein-dG, crotonaldehyde-dG and HNE-dG. The identities of these products were confirmed by comparing the HPLC retention times and ultraviolet spectrum to those of the authentic synthetic standards. Acrolein-dG and crotonaldehyde-dG were formed predominantly from docosahexaenoic acid, whereas HNE-dG adducts were formed exclusively from arachidonic acid and linoleic acid. Figure 3 shows typical HPLC chromatograms obtained from analyses of the incubation mixture with linoleic acid. The quantities of HNE-dG adducts formed by arachidonic acid and linoleic acid were 20.6 and 3.5 nmol, respectively, whereas HNE-dG adducts were not detected in the reaction with docosahexaenoic acid; 5.4 and 0 nmol (not detectable) of acrolein-dG and crotonaldehyde-dG were obtained from linoleic acid, and 54.0 and 20.2 nmol were obtained from docosahexaenoic acid, respectively. Interestingly, acrolein-dG3, the major acrolein-dG isomer detected *in vivo*, was also the

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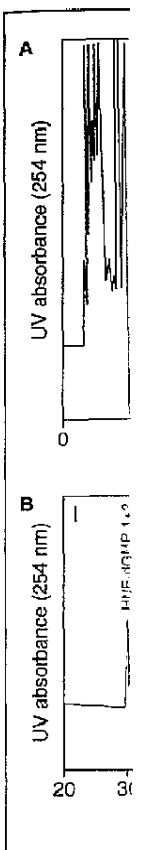

Figure 3. High-
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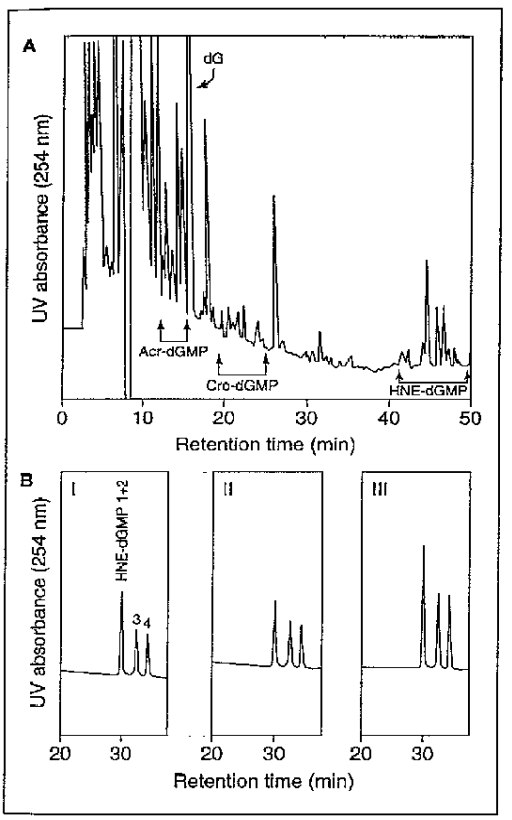


Figure 3. High-performance liquid chromatography of the incubation mixture from a reaction with linoleic acid

In (A), the chromatogram indicates the fractions corresponding to acrolein–deoxyguanosine (Acr-dG), crotonaldehyde–deoxyguanosine (Cro-dG) and *trans*-4-hydroxy-2-nonenenal–deoxyguanosine (HNE-dG) adducts; (B), chromatograms from the analysis of (i) the collected peaks corresponding to HNE-dG adducts in (A), (ii) the standard HNE-dG adducts and (iii) the HNE-dG adducts plus the standards.

Effect of depletion of reduced glutathione on acrolein–deoxyguanosine and crotonaldehyde–deoxyguanosine levels in liver DNA

Reduced glutathuone (GSH) is an endogenous antioxidant and an effective scavenger of enals. The dual function of GSH suggests that sustained depletion of this substance in tissues could increase the levels of acrolein-dG and crotonaldehyde-dG in tissue DNA, if these adducts are derived from enals generated by lipid peroxidation. We examined the effect of GSH depletion caused by L-buthionine (S,R)-sulfoximine (BSO) on the levels of these adducts in liver DNA from male Fischer 344 rats (Nath *et al.*, 1997). A group of five male rats was given BSO (30 mmol/L) in drinking-water for three weeks, and a group of eight rats was given water only. The average body weight in the BSO group was about 15% lower than that in the control group at termination. At the end of treatment, the liver from each animal was immediately processed for GSH and cyst(e)ine determination by HPLC with an electrochemical detector (Kleinman & Richie, 1995), and DNA was isolated. The hepatic GSH levels were reduced by more than 80% in the BSO-treated rats, from 4.43 to 0.72 μmol/g tissue. The mean levels of acrolein-dG3, crotonaldehyde-dG1 and crotonaldehyde-dG2 were 1.18 ± 1.03, 3.12 ± 3.26 and 2.50 ± 2.59 μmol/mol guanine in the group given BSO and 0.57 ± 0.25, 0.15 ± 0.18 and 0.16 ± 0.22 μmol/mol guanine in the control group, respectively. These increases are approximately twofold for acrolein-dG ($p = 0.17$) and 15–21-fold for crotonaldehyde-dG ($p < 0.05$).

The differential increase in acrolein-dG and crotonaldehyde-dG observed in the BSO-treated animals is intriguing. It could be due to various factors, including differences in the reactivity of acrolein and crotonaldehyde towards GSH and DNA or stability and repair of these adducts. It should be noted that the treated animals had significantly lowered body weights. These effects of BSO were unexpected, since the same dose did not affect the body weights of mice even though, on a body-weight basis, the mice were exposed to a higher dose of BSO than rats (Sun *et al.*, 1985). Nonetheless, this study demonstrates that tissue GSH may play an important role in protecting DNA from cyclic

adduction by enals; it also supports the endogenous origin of acrolein-dG and crotonaldehyde-dG.

Detection of acrolein–deoxyguanosine and crotonaldehyde–deoxyguanosine in human oral tissue DNA and their increased levels in smokers

We recently analysed human oral tissue DNA for acrolein-dG and crotonaldehyde-dG. Since smokers generally have an extra oxidative burden due to their exposure to cigarette smoke, we investigated whether the cyclic propano-dG adduct levels are higher in the oral tissues of smokers than in those of nonsmokers (Nath *et al.*, 1998). The gingival tissue DNA from 11 smokers (four men and seven women aged 30–58 years) and 12 nonsmokers (eight men and four women aged 21–66 years old) were thus analysed by the ^{32}P-postlabelling–HPLC method. We found that the mean acrolein-dG levels (μmol/mol guanine) in smokers were significantly higher than those in nonsmokers: 1.36 ± 0.90 in smokers vs. 0.46 ± 0.26 in nonsmokers (p = 0.003). The mean crotonaldehyde-dG1 levels in smokers and nonsmokers were 0.53 ± 0.44 and 0.06 ± 0.07, respectively, corresponding to an 8.8-fold increase in smokers (p = 0.0015). The levels of crotonaldehyde-dG2 were also increased, by 5.5-fold, in smokers as compared with nonsmokers, from 0.31 ± 0.40 to 1.72 ± 1.26 μmol/mol guanine (p = 0.0014). Furthermore, the total level of cyclic adducts (acrolein-dG and crotonaldehyde-dG) in smokers was 4.4-fold greater than in nonsmokers (p = 0.0003). These results are summarized in Figure 4.

The increases in acrolein-dG and crotonaldehyde-dG levels in smokers' gingival DNA observed in this study could be due to stimulated production of endogenous enals *via* lipid peroxidation caused by the oxidants in cigarette smoke and/or direct exposure to acrolein and crotonaldehyde in the smoke. The pro-oxidant state of smokers has previously been characterized by higher plasma levels of malondialdehyde, lower GSH levels and increased 8-hydroxydeoxyguanine levels in leukocytes and lung DNA (Bridges *et al.*, 1993; Hulea *et al.*, 1995; Asami *et al.*, 1996; Yarborough *et al.*, 1996). The plasma GSH levels returned to normal when smoking was stopped

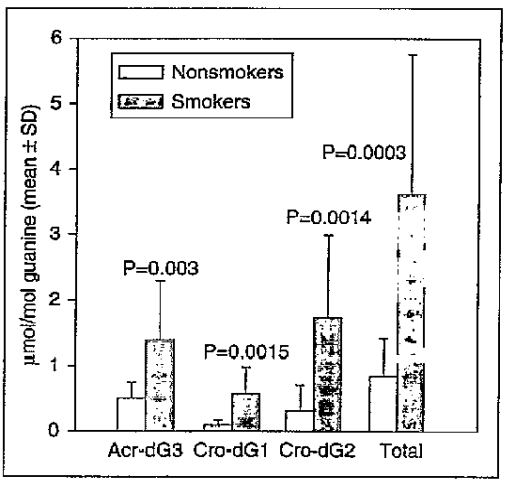


Figure 4. Levels of cyclic adducts in the oral tissue DNA of smokers and nonsmokers

Acr-dG, acrolein–deoxyguanosine; Cro-dG, crotonaldehyde–deoxyguanosine

(Lane *et al.*, 1996). As discussed above, the depletion of hepatic GSH by treatment with BSO caused a dramatic increase in the 1,N^2-propano-dG adduct levels in rat liver DNA. Interestingly, in that study, the increases in crotonaldehyde-dG in the BSO-treated rats were much more pronounced than those of acrolein-dG, a result similar to that obtained in the study in smokers. These observations lend further support to endogenous formation of acrolein-dG and crotonaldehyde-dG in smokers.

1,N^2-Propanodeoxyguanosine adducts in mutagenesis and carcinogenesis

The identification of enal-derived 1,N^2-propano-dG adducts as endogenous DNA lesions in rodent and human tissues has raised questions about their roles in mutagenesis and carcinogenesis. Although there is as yet no direct proof for their involvement in carcinogenesis, a number of studies have provided suggestive evidence.

Acrolein and crotonaldehyde are known to induce revertants in the *Salmonella* tester strains TA100 and TA104 (Marnett *et al.*, 1985), whereas

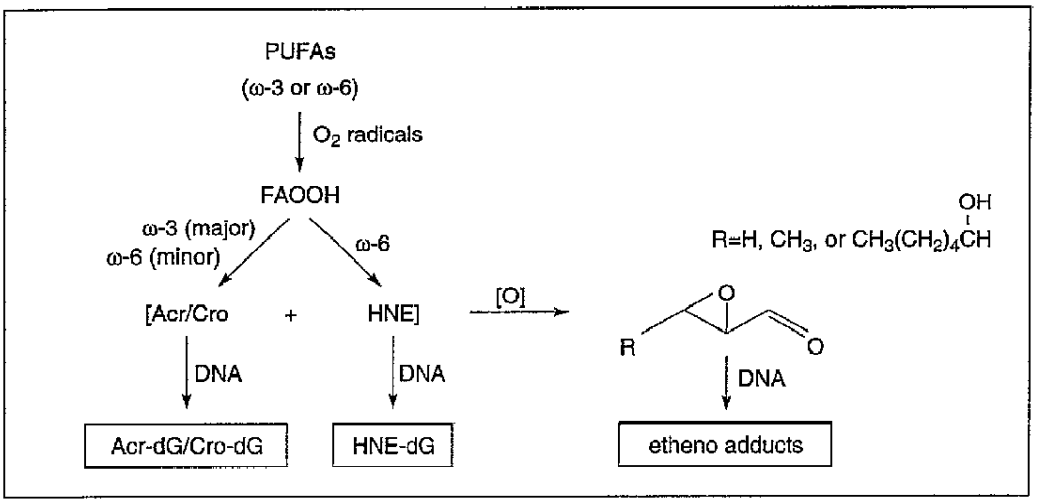
HNE was inactive in these strains (Chung *et al.*, 1993), although it induced mutation in V79 Chinese hamster cells (Cajelli *et al.*, 1987). We previously examined the mutagenicity of acrolein-dG in an immunoassay developed for acrolein-dG (Foiles *et al.*, 1989). Formation of this adduct in DNA from *S. typhimurium* tester strains TA100 and TA104 exposed to acrolein was analysed under conditions in which revertants are induced. Acrolein-dG3 was detected in a dose-dependent manner in both strains. The mutagenicity of acrolein was also measured in these strains in a liquid preincubation assay. The correlation between acrolein-dG3 levels and revertants/plate in TA100, which contains GC base pairs at the site of reversion, suggests that acrolein-dG3 is a promutagenic lesion. Acrolein also induced revertants in TA104, which has AT pairs at the site of reversion, suggesting that the adenine adducts may be involved in the mutagenicity in this strain.

Although the mutation characteristics of the etheno adducts that have been detected *in vivo*, such as 1,N^6-ethenodA, N^2,3-ethenodG and 3,N^4-ethenodC have been investigated in studies of site-specific mutagenesis (Cheng *et al.*, 1991; Palejwala *et al.*, 1991; Basu *et al.*, 1993; Pandya & Moriya, 1996), no information is available on the mutagenic property of acrolein-dG, crotonaldehyde-dG and HNE-dG. A model 1,N^2-propano-dG adduct without the hydroxy group in the propano ring has, however, been used as a substitute. This induced G→A and G→T mutations and frame-shift mutation (Benamira *et al.*, 1992; Moriya *et al.*, 1994). It is known that the outcome of studies of site-specific mutagenesis can vary with the structures of adducts, sequence context and host systems; these results, nonetheless, indicate that the model propano-dG adduct is a promutagenic lesion.

At present, there is only circumstantial evidence to support a role of acrolein-dG and crotonaldehyde-dG in carcinogenesis. The increases in adduct levels over background in rodents treated with *N*-nitrosopyrrolidine or cyclophosphamide, both of which are carcinogens known to release crotonaldehyde and acrolein, respectively, after metabolism suggest that these adducts may be important in the induction of cancer by these chemicals (Chung *et al.*, 1989; Wilson *et al.*, 1991). It should be noted, however, that other DNA alkylating agents are formed after treatment with these carcinogens, in addition to acrolein and crotonaldehyde-derived adducts. The low spontaneous liver tumour incidences in Fischer 344 rats suggests that the background propano adducts in liver may not be sufficient for tumour development during the lifetime of these animals. Further, we have shown that crotonaldehyde given in drinking-water was a weak liver carcinogen in Fischer 344 rats and gave rise to an increased formation of crotonaldehyde-dG in this tissue (Chung *et al.*, 1986, 1989). We have also shown in skin tumorigenesis in CD-1 mice and in liver tumorigenesis in newborn CD-1 mice that while the epoxide of HNE was weakly tumorigenic the parent enal was inactive in both models (Chung *et al.*, 1993). These results suggest that enals are likely to be weak tumour initiating agents. It is tempting to speculate, however, that the propano dG adducts may be important in the promotion of tumorigenesis, as this process is known to be associated with stimulated oxidative conditions.

Conclusion

The studies described here provide several lines of evidence that support the importance of enals generated by lipid peroxidation as sources for propano adducts in tissue DNA. We have shown that the formation of short-chain or long-chain propano adducts is likely to be dependent on the type of polyunsaturated fatty acids. On the basis of this information, we propose a pathway, depicted in Scheme 1, involving polyunsaturated fatty acids in the formation of acrolein-dG, crotonaldehyde-dG and HNE-dG. Further, our studies showed the presence of HNE-dG adducts in tissues. This is important because the detection of both short- and long-chain enal-derived propano adducts as background DNA lesions suggests that cyclic propano adduction is a common reaction *in vivo*, and acrolein-dG, crotonaldehyde-dG and HNE-dG may represent only a portion of these adducts in tissue DNA. Understanding of the roles of these endogenous cyclic adducts in cancer is an important and challenging area of future investigation. In this context, it is reasonable to believe that the basal levels and patterns of these promutagenic lesions in human tissues could be modi-



Scheme 1. Proposed endogenous pathway involving oxidation of polyunsaturated fatty acids (PUFAs) for the formation of acrolein–deoxyguanosine (Acr-dG), crotonaldehyde–deoxyguanosine (Cro-dG) and *trans*-4-hydroxy-2-nonenenal–deoxyguanosine (HNE-dG) adducts and the etheno adducts

The epoxidation of enals could be mediated by H_2O_2, fatty acid hydroperoxide (FAOOH) or autotoxidation (Chung *et al.*, 1996).

fied by a variety of genetic, environmental and dietary factors. These interactions are likely to profoundly influence the susceptibility of the hosts to carcinogens.

Acknowledgements

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Lipid ether

J. Nair

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Introduction

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